Evidence for synergistic activity of plant-derived essential oils against fungal pathogens of food

Farah Hossain¹, Peter Follett², Khang Dang Vu¹, Mehdi Harich¹, Stephane Salmieri¹, Monique Lacroix^{1*}

^{1*} Research Laboratories in Sciences Applied to Food, Canadian Irradiation Centre, INRS-Institute Armand-Frappier, 531 boulevard des Prairies., Laval, Québec, Canada, H7V 1B7

² USDA-ARS, U.S. Pacific Basin Agricultural Research Center, 64 Nowelo Street, Hilo, Hawaii 96720, USA

Abstract:

The antifungal activities of eight essential oils (EOs) namely basil, cinnamon, eucalyptus, mandarin, oregano, peppermint, tea tree and thyme were evaluated for their ability to inhibit growth of *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus parasiticus* and *Penicillium chrysogenum*. The antifungal activity of the EOs was assessed by the minimum inhibitory concentration (MIC) using 96-well microplate analysis. The interactions between different EO combinations were done by the checkerboard technique. The highest antifungal activity was exhibited by oregano and thyme which showed lower MIC values amongst all the tested fungi. The antifungal activity of the other EOs could be appropriately ranked in a descending sequence of cinnamon, peppermint, tea tree and basil. Eucalyptus and mandarin showed the least efficiency as they could not inhibit any of the fungal growth at 10,000 ppm. The interaction between these two EOs also showed no interaction on the tested species. A combined formulation of oregano and thyme resulted in a synergistic effect, showing enhanced efficiency against *A. flavus* and *A. parasiticus* and *P. chrysogenum*. Mixtures of peppermint and tea tree produced synergistic effect against *A. niger*. Application of a modified Gompertz model considering fungal growth parameters like maximum colony diameter, maximum specific growth

rate and lag time periods, under the various EO treatment scenarios, showed that the model could adequately describe and predict the growth of the tested fungi under these conditions.

Keywords: Essential oil, minimum inhibitory concentration (MIC), fractional inhibitory concentration (FIC), fungal growth parameter.

*Author to whom correspondence should be addressed, Telephone:+1-450-687-5010;

Fax: +1-450-686-5501;E-mail: monique.lacroix@iaf.inrs.ca

Introduction:

Fungi are regarded as one of the main concerns in food storage (Zhavah et al., 2015). *Aspergillus*, *Penicillium* and *Fusarium* are the main fungi associated with wheat, rye and corn grains under field and storage conditions (Krisch et al., 2011). Fungal growth on raw and processed foods may result in several kinds of spoilage and include textural and sensory changes, off-flavor development and odour emission, rotting and formation of pathogenic and allergenic propagules (Dellavalle et al., 2011). The deterioration of sensorial properties is often due to the production of exoenzymes during fungal growth. Once inside the food, these enzymes may continue their activities independent of the destruction or removal of the mycelium. In addition, the production of mycotoxins by fungi, in stored food commodities constitutes a serious health threat to humans and livestock. Five types of mycotoxins are deemed noxious world-wide for human health: aflatoxins, ochratoxin A, fumonisins, certain trichothecenes and zeaxalenone (Pitt et al., 2000). Long-term ingestion of these toxins as a result of eating contaminated foods has been associated with liver and kidney tumors in animals and humans. Some mycotoxins can cause auto-immune illnesses, while some are teratogenic, carcinogenic and mutagenic (Angelini et al., 2006; Garcia et al., 2009; Krisch et al., 2011). Furthermore, these toxins can accelerate lipid oxidation due to the chain reaction of free radical oxidation. Certain mycotoxins have been reported to produce free radicals which certainly impose an undesirable influence on human health (Alves-Silva et al., 2013). Hence, the development of multiple measures to prevent fungal growth, mycotoxin production and free radical generation has become a crucial aspect to combat food losses and preserve food quality during storage and transport.

The food industry has tended to reduce the use of chemical preservatives of antifungal activity due the pressure imposed by consumers and legal authorities to either completely remove these

toxic compounds or to adopt more natural alternatives for the maintenance or extension of product shelf life (Beyki et al., 2014). Essential oils (EOs) represent one of these natural additives and bear potent biological activities. In recent years, numerous *in vitro* and *in vivo* studies have reported the antifungal effects of plant EOs to control food spoilage (Gibriel et al., 2011; Mohammadi and Aminifard, 2013; Sumalan et al., 2013; Tian et al., 2012). However, the biological activity of EOs varies greatly with individual EO, depending on the chemical composition which is specific to plant parts used, method of extraction, harvesting season etc (Chaubey, 2007; Vitoratos et al., 2013).

The aim of the current research was to evaluate the inhibition activity of eight EOs against *A. niger*, *A. flavus*, *A. parasiticus* and *P. chrysogenum* by determining (i) the minimum inhibitory concentration (MIC) of the EOs and (ii) the possible synergistic effects between EO combinations, and finally (iii) antifungal activities of the volatile components resulting from the EOs to better understand the inhibitory kinetics caused by EOs on fungal growth.

2. Materials and methods

2.1 Preparation of essential oil emulsion

Basil, cinnamon, eucalyptus, mandarin, oregano, peppermint, tea tree and thyme EOs were obtained from Robert & Fils (Ghislenghien, Belgium) and stored at 4 °C prior to use. Each EO was prepared as an emulsion containing 2.5% (v/v) of EO and 2.5% (v/v) of Tween 20 (Laboratoire Mat, QC, Canada). The mixtures were homogenized for 5 min with an Ultra-Turrax homogenizator (model TP18/1059, Germany) at 20,000 rpm to obtain a colloidal suspension. The emulsions were aseptically filtered using a 0.45 µm pore size sterile filter. The mixtures

were then stored at 4 °C. The major component (provided by the manufacturer) of these EOs are presented in table 1.

2.2 Fungal inocula and assay media

Aspergillus niger (ATCC 1015), *Aspergillus flavus* (ATCC 9643), *Aspergillus parasiticus* (ATCC 16869), and *Penicillium chrysogenum* (ATCC 10106) were used for the assays. Each fungal species was grown and maintained in potato dextrose broth (PDB, Difco, Becton Dickinson) containing glycerol (10% v/v). Prior to each experiment, stock cultures were propagated through two consecutive 48 h growth cycles in PDB medium at 28°C ± 2 °C. The cultures were pre-cultured in PDA for 3 days at 28°C ± 2 °C. Conidia were isolated from the agar media using sterile saline containing 0.05% Tween 80, and the filtrate was adjusted to 1 x 10⁷ conidia/mL for broth dilution and checkerboard method and 1 x 10⁸ conidia/mL for vapor contact assay by using a microscope (Inouye et al., 2006).

2.3 Determination of the Minimum Inhibitory Concentrations (MIC) using broth dilution method

The method for determination of MIC was adopted from Turgis et al. (2012) with slight modification. All EOs were diluted in Potato Dextrose Broth (PDB) medium to obtain serial concentrations of 10000-10 ppm. A sample of 125 µl of the serially diluted EOs was pipetted into 96 wells microplate (Sarstedt, QC, Canada). Each sample well was inoculated with 15µl of fungi at a concentration of 10⁷ CFU/mL in order to obtain 1 x 10⁶ conidia/mL of final concentration. The microplate was incubated aerobically for 36 h at 28 °C. After incubation, the absorbance was measured at 590 nm using an Ultra Microplate Reader (Biotek instruments, VT, USA). Sterile PDB medium was incubated under the same condition and used as a negative

control blank. PDB medium incubated with a specific fungal species (without EO) was used as a positive control of growth. The minimum inhibitory concentration (MIC) was determined as the lowest EO concentration showing a complete growth inhibition of the tested fungal strains. This was evaluated by measuring the absorbance that is equal to the absorbance of the blank.

2.4 Assessing interaction between EO mixtures by the checkerboard method

Combination assays were evaluated based on a checkerboard procedure described by Turgis et al. (2012). The checkerboard method was performed to obtain the fractional inhibitory concentration (FIC) index of mixtures of EO combinations against each mold species. The index was calculated by adding the FIC values of EO (a) denoted by FIC_a and (b) denoted by FIC_b . The FIC_a and FIC_b values represented the fraction of the lowest concentrations of EOs and mixtures of EOs, respectively, that caused inhibition of fungal growth in the combination tests. The calculations were performed using the following equations

$$FIC_a = MIC_{a,combined} / MIC_{a,alone} \quad (1)$$

$$FIC_b = MIC_{b,combined} / MIC_{b,alone} \quad (2)$$

$$FIC = FIC_a + FIC_b \quad (3)$$

Based on the above, the FIC of an EO could be equated to the concentration which caused deactivation of the fungal species when used in combination with another EO divided by the concentration that had the same effect when used alone (Gutierrez et al., 2009; Mamoudou et al., 2010). An $FIC \leq 0.5$ was interpreted as a synergistic effect, $0.5 \leq FIC \leq 1$ represented as an additive effect, $FIC \leq 4$ represented as no interactive effect and $FIC > 4$ indicated an antagonistic effect between two tested EOs (Gutierrez et al., 2008; Krisch et al., 2011).

2.5 Vapor Contact Assays

Vapor contact assays were performed based on the method described by Inouye et al. (2006). A sample of 1 mL containing 1×10^8 of conidial suspension of the each fungal species were added to 100 mL of agar medium containing 1% peptone, 1% glucose and 1% agarose at 50 °C. A volume of 3 mL of the prepared mixture was overlaid onto the surface of hardened PDA medium (20 ml) in a Petri dish (83 mm in diameter) to prepare a double layered agar medium. Sterile filter paper (10 mm diameter) was placed at the center of the upper lid of the plate. A quantity of 10 µl of each EO was added at the center of individual paper filters. Since oregano and thyme showed a synergistic effect based on their computed FIC index, their combined vapor activity was investigated by adding 10 µL of oregano and thyme mixture (1:1 v/v) on paper filter. Sterile water was used as control. The resulting Petri dishes containing the double-layered agar medium were inverted and placed on the upper lid containing the EOs. The Petri dishes were then incubated at $28 \text{ °C} \pm 2 \text{ °C}$ for 1 week. The inhibitory diameter zone, which showed absence of growth of the test microorganisms, was measured in mm using a Traceable® Carbon Fiber Digital Caliper (Fisher Scientific). The amount of fungal growth was assessed by subtracting any clear inhibition zone produced from the internal diameter of the Petri dish. Based on the assays, the maximum colony diameter, lag time and mold growth rate were used as variables for modeling studies described in section 2.6 below.

2.6. Gompertz modeling of fungal growth:

Fungal growth modeling and growth parameters were fitted using the modified Gompertz model proposed by Char et al. (2007) and Avila-Sosa et al. (2012) using the following growth parameter relationship.

$$\ln(D_t/D_o) = A \exp\{-\exp[(v_m \cdot e/A)(\lambda - t) + 1]\} \quad (4)$$

where, D_t (cm) is the average colony diameter at time t (day), D_o (cm) is the average colony diameter at initial time, A is the maximum colony diameter achieved during the stationary phase, v_m is the maximum growth rate (1/day), λ is the lag phase (day) and $e = \exp(1)$.

2.7 Statistical Analysis

All experiments were done in triplicate. The experiments were then repeated two more times (with each treatment performed in triplicate). Fractional Inhibitory Concentration (FIC) of combined EOs and modified Gompertz model parameters against four fungal species were analyzed with the SPSS program (SPSS, Chicago, Ill.). An analysis of variance was performed using PASW Statistics Base 18 software (SPSS Inc., Chicago, IL), and comparisons of means between treatments and target fungal species were based on Duncan's multiple range tests at 5% level.

3. Results and Discussion

3.1 Minimum Inhibitory Concentrations (MIC) of EOs

The MICs obtained for the eight EOs are presented in Table 2. Based on MIC values, EOs were classified into three distinct groups corresponding to i) highly effective (312.5 ppm-5000 ppm) ii) moderately effective (5000-10,000 ppm), and iii) less effective (>10,000ppm). Among the tested EOs, thyme and oregano were found to be the most effective against all the four fungal species tested, with consistently low MIC values, followed by cinnamon. *A. niger* and *P. chrysogenum* were sensitive to oregano with a MIC of as low as 625 ppm. *A. flavus* and *A. parasiticus* showed less susceptibility to oregano bearing a MIC of 2500 ppm. Previous studies

also showed similar trends. Viuda-Martos et al. (2007) reported that oregano induced the highest degree of inhibition against *A. niger* among other tested EOs including clove and thyme.

In the present study, *A. flavus* was found to be more sensitive to thyme with a MIC of 1250 ppm, followed by oregano, with a MIC of 2500 ppm. The other tested EOs cinnamon, peppermint and tea tree showed a MIC value of 5000 ppm against *A. flavus*. The study conducted by Omidbeygi. et al. (27) also reported that thyme exhibited the highest antifungal activity against *A. flavus* followed by summer savory and clove in both *in vitro* and *in vivo* tests. In our study, *A. parasiticus* was found to be sensitive to thyme with a MIC value of 1250 ppm.

Cinnamon EOs found to be very effective with a MIC of 2500 ppm against *A. niger*, *A. parasiticus* and *P. chrysogenum* and 5000 ppm for *A. flavus*. Basil and tea tree could be grouped into the moderately effective category of EOs with MICs ranging from 5000-10,000 ppm against all tested fungi. Peppermint showed good activity against *P. chrysogenum* with a MIC of 1250 ppm but lower activity against *A. parasiticus* and *A. flavus* with MIC values of 2500 and 5000 ppm, respectively. *A. niger* showed more resistance to peppermint in comparison to the other tested fungi, exhibiting a MIC of up to 10,000 ppm. In a similar way, *P. chrysogenum* was found to be more resistant to basil and tea tree as compared to other tested EOs. Eucalyptus and mandarin did not inhibit the growth of any of the test specimens at concentrations of up to 10,000 ppm. Safaei-Ghomi and Ahd (2010) reported that eucalyptus oil, whose major component is 1, 8 cineole, did not inhibit the growth of *A. niger* in diffusion and MIC tests and showed lower degree of antimicrobial activity compared to other EOs containing limonene, α -pinene, p-cymene, and terpineol-4-ol. Cineole-containing EOs, thus, seem to display low antifungal properties unlike phenolic compounds such as carvacrol, thymol, and eugenol which display major fungicidal effects. Etherified compounds such as anethole, methyl chavicol, and

safole exhibit low fungicidal action, while monoterpene hydrocarbons such as limonene and myrcene have almost no effect.

Our findings generally showed that the EOs had antifungal activity, however each EO showed a specific pattern of activity against the fungal species tested. The variation in the fungicidal action of the different EOs used in our study may be explained by the difference in the water solubility and lipophilic properties of the EOs. Cinnamon which contains linalool as a major component, exhibited higher antifungal activity compared to basil and peppermint, which contain methyl chavicol and menthol as their main components. Linalool has an inherently higher water solubility compared to chavicol and menthol, and thus may exhibit higher antifungal activity. However, specific functional groups of EOs and the interference of membrane-associated enzyme proteins may also affect inhibitory processes of EOs (Suppakul et al., 2003).

3.2. Fractional Inhibitory Concentration (FIC) of combined EOs against fungal species

The FICs of the EO combinations tested during the current study are presented in Table 3. The highly effective oregano and thyme combination displayed a synergistic effect against all the fungi except for *A. niger*. Stević et al. (2014) reported a synergistic effect between carvacrol and thymol contained in oregano and thyme against *A. flavus*, *A. alternata*, *Penicillium sp* and *Fusarium* species. Such synergism between carvacrol and thymol has been reported against the bacteria *S. typhimurium* where a combined effect resulted in enhanced reduction of the bacterial population than when applied alone (Zhou et al., 2007). Here we report similar observations for fungal species.

The mixture of oregano and thyme showed an additive effect against *A. niger*. Thyme mixed with cinnamon, however, exhibited a synergistic effect against *A. flavus*. Combination of

oregano with cinnamon, tea tree, thyme and mint, and an individual mixture of thyme and mint resulted in synergistic effects against *P. chrysogenum*. Similarly, a combination of cinnamon and thyme, oregano and mint, oregano and thyme showed a synergistic effect against *A. flavus*. Mint mixed with tea tree showed a synergistic effect against *A. niger*. Other mixtures namely basil and mint, cinnamon and tea tree, eucalyptus and tea tree, cinnamon and eucalyptus showed additive effects against the four tested fungi.

In contrast, mandarin and eucalyptus, when combined with thyme and oregano respectively, produced no interaction and resulted in FIC values ranging from 1.2-2.5 against all the tested fungi. It should be noted that synergism between antimicrobial agents such as carvacrol and some hydrocarbons monoterpenes (such as α -pinene, camphene, myrcene, α -terpinene and p-cymene) has been reported previously, which on a standalone basis, typically display low antimicrobial properties (De Azeredo et al., 2011; Ultee et al., 2000). Such synergism may stem from the capacity of hydrocarbons to interact with the microbial cell membrane thereby facilitating the penetration of carvacrol into the cell (De Azeredo et al., 2011; Ultee et al., 2002; Ultee et al., 2000). Synergy is not only influenced by major compounds of essential oils alone, since minor components may have a more critical role to this effect with the activity of the main components being modulated by other minor molecules (Sahaf and Moharramipour, 2008). Most of the antimicrobial activity of EOs appears to be derived from oxygenated terpenoids, particularly phenolic terpenes, phenylpropanoids and alcohols. Other constituents such as hydrocarbons that typically show low activities can be used in combinations to increase their bioactivities (Bassolé and Juliani, 2012). Hence, in the present study, combination of some particular oils showed additive and synergistic activity when combined, due to the combined activities of two or more components of EOs. Such increased fungistatic activity would have the

advantage in pre- and post- harvest protection because pathogens cannot easily acquire resistance to multiple components of the two or more EOs (Stević et al., 2014).

3.3. Gompertz modeling of fungal growth

The variation in colony diameter of the four fungal species under different EO treatments is presented in Figures 1-4. The modified Gompertz model developed by Avila-Sosa et al. (2012) was used to compute the fungal growth in presence of the EOs, and the predictions of the model parameters were compared with the actual observed experimental data. Model outputs describing the lag time, maximum specific growth rate, and maximum mold growth during the stationary phase, are shown in Table 4. The proposed model adequately described the growth curves exhibited by the fungi in presence of the eight EOs, and the mixture of oregano and thyme. The maximum colony diameter of mold growth achieved during the stationary phase (A), the maximum exponential growth rate (v_m) and the lag time (λ) values obtained from the growth curves provided useful quantitative comparison among the various EOs tested against the four fungal species. Concurrently, the modified Gompertz model was used to fit the data to predict the model parameters of the fungal species. No significant difference ($P>0.05$) was found between the Gompertz predicted model growth parameter values and the actual observed experimental values. The results of the vapor assays were used to compute the model parameters. In this context, oregano, and thyme induced the highest growth inhibition for the four tested fungal species. Oregano and thyme also had the highest negative impact on the growth of the tested molds by decreasing their maximum growth rates (v_m). Eucalyptus and mandarin were found to be the least effective EOs in limiting the maximum growth rate of the fungi. Cinnamon and tea tree significantly ($P>0.05$) reduced the growth rate of *P. chrysogenum*. Regarding the lag phase (λ), thyme was found to be the most effective against *A. niger* and *A. parasiticus*,

increasing their lag time up to 3 days, followed by *P. chrysogenum* (2.6 d) and *A. flavus* (2.1 d). Oregano was found to be the most effective against *P. chrysogenum* in extending its lag phase to 2.83 d. As shown in table 4 and figure 5, a combination of oregano and thyme was very effective against all the four tested fungal species; this combination significantly ($P>0.05$) limited the colony diameter and the maximum specific growth rate of the fungal species and extended their lag times. These findings provide valuable insights into how the EOs exerted their antifungal activities by altering the growth kinetics of the tested fungi. Avila-Sosa et al. (2010) demonstrated how growth parameters derived from a modified Gompertz model affected the growth kinetics of *A. niger* in presence of the Mexican oregano added to amaranth, chitosan and starch edible films. The current research extends the observations made during previous studies by examining the growth parameters of other fungal species for a wider variety of EOs and combinations.

Conclusions:

The present research highlights the potential antifungal effects of eight EOs, alone and in combination, against four mold. Based on MIC assays, the EOs were broadly grouped as having high, moderate and low effective groups with oregano and thyme showing the highest antifungal activity. Checkerboard analyses showed that some EO combinations displayed synergistic effects as was the case for oregano and thyme, while others such as mandarin and eucalyptus mixed with thyme and oregano respectively, showed no interaction at all. Studying the effect of the EOs against the fungi using the modified Gompertz model provided valuable insights on their growth parameters. In general, the EOs were effective in reducing the colony diameter and exponential growth rate while increasing the lag time of the fungi tested. Further research on the antifungal activity of EOs and their main components are required together with a better understanding of

298 the mode of action in order to evaluate their usefulness in extending the shelf-life of packaged
299 foods.

300 **Acknowledgement**

301 We are thankful to the United States Department of Agriculture (USDA) and the Natural
302 Sciences and Engineering Research Council of Canada (NSERC) for their support and funding.

303 The authors are also thankful to Ministère de l'Économie, de l'Innovation et des Exportations,
304 Quebec.

References

- Alves-Silva, J.M., Dias dos Santos, S.M., Pintado, M.E., Pérez-Álvarez, J.A., Fernández-López, J., Viuda-Martos, M., 2013. Chemical composition and in vitro antimicrobial, antifungal and antioxidant properties of essential oils obtained from some herbs widely used in Portugal. *Food Control* 32, 371-378.
- Angelini, P., Pagiotti, R., Menghini, A., Vianello, B., 2006. Antimicrobial activities of various essential oils against foodborne pathogenic or spoilage moulds. *Annals of microbiology* 56, 65-69.
- Avila-Sosa, R., Palou, E., Jiménez Munguía, M.T., Nevárez-Moorillón, G.V., Navarro Cruz, A.R., López-Malo, A., 2012. Antifungal activity by vapor contact of essential oils added to amaranth, chitosan, or starch edible films. *International Journal of Food Microbiology* 153, 66-72.
- Avila-Sosa, R., Hernández-Zamoran, E., López-Mendoza, I., Palou, E., Munguía, M.T.J., Nevárez-Moorillón, G.V., López-Malo, A., 2010. Fungal inactivation by Mexican oregano (*Lippia berlandieri* Schauer) essential oil added to amaranth, chitosan, or starch edible films. *Journal of Food Science* 75, M127-M133.
- Bassolé, I.H.N., Juliani, H.R., 2012. Essential oils in combination and their antimicrobial properties. *Molecules* 17, 3989-4006.
- Beyki, M., Zhavah, S., Khalili, S.T., Rahmani-Cherati, T., Abollahi, A., Bayat, M., Tabatabaei, M., Mohsenifar, A., 2014. Encapsulation of *Mentha piperita* essential oils in chitosan-cinnamic acid nanogel with enhanced antimicrobial activity against *Aspergillus flavus*. *Industrial Crops and Products* 54, 310-319.
- Char, C.D., Guerrero, S.N., Alzamora, S.M., 2007. Growth of *Eurotium chevalieri* in milk jam: influence of pH, potassium sorbate and water activity. *Journal of Food Safety* 27, 1-16.
- Chaubey, M.K., 2007. Toxicity of essential oils from *Cuminum cyminum* (Umbelliferae), *Piper nigrum* (Umbelliferae) against stored-product beetle *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae). *Electronic Journal of Environmental, Agricultural and Food Chemistry* 6, 1719-1727.
- De Azeredo, G.A., Stamford, T.L.M., Nunes, P.C., Gomes Neto, N.J., De Oliveira, M.E.G., De Souza, E.L., 2011. Combined application of essential oils from *Origanum vulgare* L. and *Rosmarinus officinalis* L. to inhibit bacteria and autochthonous microflora associated with minimally processed vegetables. *Food Research International* 44, 1541-1548.
- Dellavalle, P.D., Cabrera, A., Alem, D., Larrañaga, P., Ferreira, F., Rizza, M.D., 2011. Antifungal activity of medicinal plant extracts against phytopathogenic fungus *Alternaria* spp. *Chilean Journal of Agricultural Research* 71, 231-239.
- Garcia, D., Ramos, A.J., Sanchis, V., Marín, S., 2009. Predicting mycotoxins in foods: a review. *Food Microbiology* 26, 757-769.
- Gibriel, Y.A.Y., Hamza, A.S., Gibriel, A.Y., Mohsen, S.M., 2011. *In vivo* effect of mint (*Mentha viridis*) essential oil on growth and aflatoxin production by *Aspergillus flavus* isolated from stored corn. *Journal of Food Safety* 31, 445-451.
- Gutierrez, J., Barry-Ryan, C., Bourke, P., 2008. The antimicrobial efficacy of plant essential oil combinations and interactions with food ingredients. *International Journal of Food Microbiology* 124, 91-97.
- Gutierrez, J., Barry-Ryan, C., Bourke, P., 2009. Antimicrobial activity of plant essential oils using food model media: efficacy, synergistic potential and interactions with food components. *Food Microbiology* 26, 142-150.
- Inouye, S., Uchida, K., Maruyama, N., Yamaguchi, H., Abe, S., 2006. A novel method to estimate the contribution of the vapor activity of essential oils in agar diffusion assay. *Japanese Journal of Medical Mycology* 47, 91-98.

Krisch, J., Rentsenkhand, T., Vágvölgyi, C., 2011. Essential oils against yeast and moulds causing food spoilage. *Science against microbial pathogens: Communicating current research and technological advances microbiology*, 1135-1142.

Mamoudou, H., Aline Lamien-Meda, A., Bayala, B., Tirogo, S., Chlodwig Franz, C., Johannes Novak, J., Nebié, R.C., Charles, R., BASSOLE, I.N., 2010. Composition and antimicrobial activities of *Lippia multiflora* Moldenke, *Mentha x piperita* L. and *Ocimum basilicum* L. essential oils and their major monoterpene alcohols alone and in combination. *Molecules* 15, 7825-7839.

Mohammadi, S., Aminifard, M.H., 2013. Inhibition of grey mould in vitro and in vivo with essential oil of fennel (*Foeniculum vulgare* L.). *Journal of Central European Agriculture* 14, 420-431.

Pitt, J., Basilico, J., Abarca, M., Lopez, C., 2000. Mycotoxins and toxigenic fungi. *Medical Mycology* 38, 41-46.

Safaei-Ghomi, J., Ahd, A.A., 2010. Antimicrobial and antifungal properties of the essential oil and methanol extracts of *Eucalyptus largiflorens* and *Eucalyptus intertexta*. *Pharmacognosy Magazine* 6, 172.

Sahaf, B.Z., Moharramipour, S., 2008. Fumigant toxicity of *Carum copticum* and *Vitex pseudo-negundo* essential oils against eggs, larvae and adults of *Callosobruchus maculatus* *Jornal of Pest Science* 81, 213-220.

Stević, T., Berić, T., Šavikin, K., Soković, M., Gođevac, D., Dimkić, I., Stanković, S., 2014. Antifungal activity of selected essential oils against fungi isolated from medicinal plant. *Industrial Crops and Products* 55, 116-122.

Sumalan, R.-M., Alexa, E., Poiana, M.-A., 2013. Assessment of inhibitory potential of essential oils on natural mycoflora and Fusarium mycotoxins production in wheat. *chemistry central*.

Suppakul, P., Miltz, J., Sonneveld, K., Bigger, S.W., 2003. Active Packaging Technologies with an Emphasis on Antimicrobial Packaging and its Applications. *Journal of food science* 68, 408-420.

Tian, J., Ban, X., Zeng, H., He, J., Chen, Y., Wang, Y., 2012. The Mechanism of Antifungal Action of Essential Oil from Dill (*Anethum graveolens* L.) on *Aspergillus flavus* *PloS One* 7, 1-10.

Turgis, M., Khanh, D.V., Dupont, C., Lacroix, M., 2012. Combined antimicrobial effect of essential oils and bacteriocins against foodborne pathogens and food spoilage bacteria. *Food Research International* 48, 696-702.

Ultee, A., Bennik, M., Moezelaar, R., 2002. The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus*. *Applied and Environmental Microbiology* 68, 1561-1568.

Ultee, A., Slump, R., Steging, G., Smid, E., 2000. Antimicrobial activity of carvacrol toward *Bacillus cereus* on rice. *Journal of Food Protection* 63, 620-624.

Vitoratos, A., Bilalisd, I., Karkanis, A., Efthimiadou, A., 2013. Antifungal Activity of Plant Essential Oils Against *Botrytis cinerea*, *Penicillium italicum* and *Penicillium digitatum*. *Not Bot Horti Agrobo* 44, 86-92.

Viuda-Martos, M., Ruiz-Navajas, Y., Fernández-López, J., Pérez-Álvarez, J.A., 2007. Antifungal activities of thyme, clove and oregano essential oils. *Journal of food safety* 27, 91-101.

Zhaveh, S., Mohsenifar, A., Beiki, M., Khalili, S.T., Abdollahi, A., Rahmani-Cherati, T., Tabatabaei, M., 2015. Encapsulation of *Cuminum cyminum* essential oils in chitosan-caffeic acid nanogel with enhanced antimicrobial activity against *Aspergillus flavus*. *Industrial Crops and Products* 69, 251-256.

Zhou, F., Ji, B., Zhang, H., Jiang, H., Yang, Z., Li, J., Li, J., Yan, W., 2007. The antibacterial effect of cinnamaldehyde, thymol, carvacrol and their combinations against the foodborne pathogen *Salmonella typhimurium*. *Journal of Food Safety* 27, 124-133.

397 **Table 1: List of EOs and their major active components**

Essential oil	Latin name	Major components
Basil	<i>Ocimum basilicum</i>	Estragole, Eugenol, linalool
Cinnamon	<i>Cinnamomum zeylandicum</i>	Linalool, trans-cinnamaldehyde
Eucalyptus	<i>Eucalyptus Globus</i>	1,8 cineole
Mandarin	<i>Citrus reticulata</i>	Limonene, γ terpinene
Oregano	<i>Origanum vulgare</i>	Carvacrol, thymol
Peppermint	<i>Mentha piperita</i>	Menthol, menthone,
Tea tree	<i>Melaleuca alternifolia</i>	Terpinol
Thyme	<i>Thymus vulgaris</i>	Thymol, carvacrol, γ -terpinene

398
399
400
401
402
403
404
405
406
407
408

Table: 2. Minimum Inhibitory Concentration (MIC) (ppm) of tested EOs against 4 fungal species after 36 h of incubation using broth dilution method.

Essential oil	Minimum inhibitory concentration (MIC) (ppm)			
	<i>A. niger</i>	<i>A. flavus</i>	<i>A. parasiticus</i>	<i>P. chrysogenum</i>
Basil	10000	10000	5000	10000
Cinnamon	2500	5000	2500	2500
Eucalyptus	10000	> 10000	>10000	> 10000
Mandarin	> 10000	10000	10000	> 10000
Oregano	625	2500	2500	625
Peppermint	10000	5000	2500	1250
Tea tree	10000	5000	5000	10000
Thyme	1250	1250	1250	312.5

Table 3. Fractional Inhibitory Concentration (FIC) of combined EOs against target fungal species

EO Mixtures	<i>A. niger</i>		<i>A. flavus</i>		<i>A. parasiticus</i>		<i>P. chrysogenum</i>	
	FIC activity	Effect	FIC activity	Effect	FIC activity	Effect	FIC activity	Effect
Basil+ Cinnamon	2.00± 0.28 ^{efgh,B}	I	2.16± 0.16 ^{ijk,B}	I	4.00± 0.16 ^{h,C}	I	0.83± 0.07 ^{abc,A}	AD
Basil + Eucalyptus	2.17± 0.20 ^{efgh,A}	I	3.16± 0.28 ^{l,A}	I	3.33± 0.33 ^{g,B}	I	2.33± 0.44 ^{fgh,A}	I
Basil+ Mandarin	2.50± 0.28 ^{hi,A}	I	2.00± 0.21 ^{hijk,A}	I	2.33± 0.18 ^{f,A}	I	2.67± 0.16 ^{h,A}	I
Basil + Oregano	1.00± 0.1 ^{abcd,A}	AD	1.50± 0 ^{efghi,AB}	I	1.25± 0.13 ^{cd,AB}	I	1.00± 0.00 ^{bc,A}	AD
Basil+ Peppermint	0.83± 0.08 ^{abc,AB}	AD	0.83± 0 ^{abcde,AB}	AD	1.00± 0.12 ^{cd,A}	AD	0.58± 0.06 ^{ab,A}	AD
Basil + Tea tree	1.67± 0.33 ^{defg,A}	I	1.33± 0.12 ^{defgh,A}	I	1.00± 0.16 ^{abcd,A}	I	1.00± 0.00 ^{bc,A}	AD
Basil + Thyme	1.41± 0.08 ^{bcd,e,A}	I	0.99± 0.12 ^{abcde, A}	AD	1.04± 0.11 ^{abcd,A}	I	1.25± 0.12 ^{cd,A}	I
Cinnamon + Eucalyptus	1.00± 0.13 ^{abcd,A}	AD	0.70± 0 ^{abcd,A}	AD	0.99± 0.10 ^{abcd,A}	AD	0.83± 0.04 ^{abc,A}	AD
Cinnamon + Mandarin	2.33± 0.16 ^{gh,C}	I	1.58± 0.12 ^{fghij,B}	I	1.16± 0.16 ^{bcd,A}	I	2.00± 0.28 ^{ef,BC}	I
Cinnamon + Oregano	4.00± 0.14 ^{kl,D}	A	3.16± 0.16 ^{l,C}	I	2.00± 0.18 ^{ef,B}	I	0.33± 0.04 ^{a,A}	S
Cinnamon + Peppermint	4.16± 0.16 ^{ghi,B}	A	1.83± 0.16 ^{ghij,B}	I	2.35± 0.25 ^{f,B}	I	1.00± 0.12 ^{bc,A}	AD
Cinnamon + Tea tree	1.00± 0.12 ^{abcd,B}	AD	0.54± 0.11 ^{abc,A}	AD	0.54± 0.08 ^{abcd,A}	AD	0.75± 0.06 ^{abc,AB}	AD
Cinnamon + Thyme	2.00± 0.16 ^{fgh,B}	I	0.41± 0 ^{a,A}	S	1.00± 0.00 ^{abcd,B}	AD	1.00± 0.00 ^{bc,B}	AD
Eucalyptus + Mandarin	2.33± 0.33 ^{ghi,A}	I	2.30± 0.12 ^{jk,A}	I	1.00± 0.00 ^{ef,A}	I	1.00± 0.00 ^{gh,A}	I
Eucalyptus + Oregano	1.33± 0.08 ^{bcd,e, A}	I	1.50± 0.12 ^{fghij,A}	I	2.08± 0.16 ^{ef,A}	I	2.50± 0.16 ^{gh,A}	I
Eucalyptus + Peppermint	1.50± 0 ^{cdef,C}	I	1.25± 0.12 ^{cdefg,B}	I	2.41± 0.23 ^{f,B}	I	1.25± 0.07 ^{cd,A}	I
Eucalyptus + Tea tree	1.00± 0.16 ^{abcd,C}	AD	0.79± 0 ^{abcde,B}	AD	1.20± 0.14 ^{bcd,B}	AD	0.62± 0.00 ^{ab,A}	AD
Eucalyptus + Thyme	2.17± 0.17 ^{fgh,A}	I	1.67± 0.16 ^{fghij,A}	I	0.60± 0.02 ^{abc,A}	AD	1.00± 0.0 ^{bc,C}	AD
Mandarin + Oregano	3.00± 0 ^{ij,B}	I	2.67± 0.10 ^{kl,B}	I	2.00± 0.28 ^{ef,A}	I	2.16± 0.18 ^{ef,A}	I
Mandarin + Peppermint	2.33± 0.17 ^{hi,C}	I	1.50± 0.12 ^{efghi,B}	I	1.33± 0.16 ^{d,A}	I	1.66± 0.16 ^{de,A}	I
Mandarin + Tea tree	3.50± 0.50 ^{jk,B}	I	1.50± 0.12 ^{efghi,B}	I	1.00± 0.00 ^{abcd,B}	AD	1.00± 0.01 ^{bc,A}	AD
Mandarin + Thyme	2.67± 0.16 ^{hi,C}	I	2.00± 0.18 ^{hijk,A}	I	2.00± 0.19 ^{ef,A}	I	1.25± 0.13 ^{cd,A}	I
Oregano + Peppermint	0.92± 0.08 ^{ab,B}	AD	1.83± 0.14 ^{ghij,B}	I	1.25± 0.14 ^{cd,A}	I	1.66± 0.11 ^{de,A}	I
Oregano + Tea tree	1.00± 0.10 ^{abcd,A}	AD	0.50± 0.06 ^{ab, A}	S	0.99± 0.10 ^{abcd,B}	AD	0.50± 0.00 ^{ab,A}	S
Oregano + Thyme	1.00± 0.10 ^{abcd,A}	AD	3.00± 0.12 ^{l,B}	I	2.33± 0.26 ^{f,A}	I	0.79± 0.00 ^{abc,A}	AD
Peppermint + Tea tree	0.75± 0.16 ^{ab,A}	AD	0.47± 0.04 ^{ab, A}	S	0.41± 0.00 ^{a,A}	S	0.41± 0.00 ^{a,A}	S
Peppermint + Thyme	0.43± 0.06 ^{a,A}	S	1.16± 0.12 ^{bcd,efg, C}	I	0.54± 0.04 ^{ab,A}	AD	0.83± 0.01 ^{abc,B}	AD
Tea tree + Thyme	0.79± 0.11 ^{abc,A}	AD	0.79± 0 ^{abcde,A}	AD	1.50± 0.19 ^{de,B}	I	0.33± 0.00 ^{a,A}	S
	1.16± 0.08 ^{bcd,C}	I	1.00± 0.1 ^{abcde,BC}	AD	0.83± 0.09 ^{abcd,AB}	AD	0.75± 0.00 ^{abc,A}	AD

441 Activity: FIC≤0.5: synergic effect (S); 0.5<FIC≤1: additive effect (AD); 1<FIC≤4: no interactive effect (I); FIC>4: antagonistic effect (A)

442 Values are means ± standard error. Within each row, means with the same uppercase letter are not significantly different (P > 0.05). Within each column

443 means with the same lowercase letter are not significantly different (P > 0.05).

Table 4. Modified Gompertz model parameters (means $\pm$ standard error) derived for four fungal species subjected to eight EOs and one mixture of EO through vapor assay.

EO	<i>A. niger</i>			<i>A. flavus</i>			<i>A. parasiticus</i>			<i>P. chrysogenum</i>		
	A (cm)	v_m (d ⁻¹)	λ (d)	A (cm)	v_m (d ⁻¹)	λ (d)	A (cm)	v_m (d ⁻¹)	λ (d)	A (cm)	v_m (d ⁻¹)	λ (d)
Basil	2.12 $\pm$ 0.0 ^d	2.20 $\pm$ 0.0 ^c	1.00 $\pm$ 0.0 ^b	2.12 $\pm$ 0.0 ^c	2.21 $\pm$ 0.1 ^e	1.00 $\pm$ 0.1 ^b	2.12 $\pm$ 0.0 ^d	2.75 $\pm$ 0.0 ^e	0.50 $\pm$ 0.0 ^a	2.12 $\pm$ 0.0 ^c	2.17 $\pm$ 0.0 ^d	1.00 $\pm$ 0.0 ^a
Cinnamon	2.12 $\pm$ 0.0 ^d	2.10 $\pm$ 0.1 ^c	1.00 $\pm$ 0.0 ^b	2.12 $\pm$ 0.0 ^c	1.86 $\pm$ 0.2 ^d	1.00 $\pm$ 0.0 ^b	2.12 $\pm$ 0.0 ^d	2.32 $\pm$ 0.2 ^d	1.00 $\pm$ 0.0 ^b	2.12 $\pm$ 0.0 ^c	1.74 $\pm$ 0.2 ^c	1.00 $\pm$ 0.0 ^a
Eucalyptus	2.12 $\pm$ 0.0 ^d	2.63 $\pm$ 0.1 ^d	0.50 $\pm$ 0.0 ^a	2.12 $\pm$ 0.0 ^c	3.45 $\pm$ 0.3 ^f	0.50 $\pm$ 0.1 ^a	2.12 $\pm$ 0.0 ^d	4.34 $\pm$ 0.3 ^g	0.50 $\pm$ 0.0 ^a	2.12 $\pm$ 0.0 ^c	4.56 $\pm$ 0.1 ^f	0.50 $\pm$ 0.0 ^a
Mandarin	2.12 $\pm$ 0.0 ^d	4.02 $\pm$ 0.1 ^e	0.50 $\pm$ 0.0 ^a	2.12 $\pm$ 0.0 ^c	4.24 $\pm$ 0.1 ^g	0.50 $\pm$ 0.1 ^a	2.12 $\pm$ 0.0 ^d	3.97 $\pm$ 0.2 ^f	0.50 $\pm$ 0.0 ^a	2.12 $\pm$ 0.0 ^c	2.90 $\pm$ 0.1 ^e	1.00 $\pm$ 0.1 ^a
Oregano	1.47 $\pm$ 0.1 ^c	0.72 $\pm$ 0.1 ^a	2.00 $\pm$ 0.0 ^c	1.66 $\pm$ 0.1 ^b	0.73 $\pm$ 0.0 ^a	2.00 $\pm$ 0.1 ^c	1.43 $\pm$ 0.1 ^b	0.58 $\pm$ 0.0 ^b	2.00 $\pm$ 0.3 ^c	1.41 $\pm$ 0.2 ^b	0.64 $\pm$ 0.0 ^b	2.83 $\pm$ 0.2 ^c
Peppermint	2.12 $\pm$ 0.0 ^d	1.55 $\pm$ 0.1 ^b	1.00 $\pm$ 0.0 ^b	2.12 $\pm$ 0.0 ^c	1.65 $\pm$ 0.1 ^c	1.00 $\pm$ 0 ^b	2.12 $\pm$ 0.0 ^d	2.12 $\pm$ 0.0 ^d	1.00 $\pm$ 0 ^b	2.12 $\pm$ 0.0 ^c	1.82 $\pm$ 0.1 ^e	1.00 $\pm$ 0.1 ^a
Tea tree	2.12 $\pm$ 0.0 ^d	1.45 $\pm$ 0.1 ^b	2.00 $\pm$ 0.0 ^c	2.12 $\pm$ 0.0 ^c	1.47 $\pm$ 0.1 ^b	1.00 $\pm$ 0 ^b	2.12 $\pm$ 0.0 ^d	2.24 $\pm$ 0.1 ^d	2.00 $\pm$ 0 ^c	2.12 $\pm$ 0.0 ^c	1.90 $\pm$ 0.1 ^c	2.00 $\pm$ 0.0 ^b
Thyme	1.21 $\pm$ 0.0 ^b	0.60 $\pm$ 0.1 ^a	3.00 $\pm$ 0.2 ^d	1.46 $\pm$ 0.1 ^a	0.62 $\pm$ 0.0 ^a	2.16 $\pm$ 0.1 ^c	1.63 $\pm$ 0.0 ^c	0.82 $\pm$ 0.0 ^c	3.00 $\pm$ 0.2 ^d	1.44 $\pm$ 0.1 ^b	0.70 $\pm$ 0.0 ^b	2.66 $\pm$ 0.3 ^c
Oregano + Thyme	1.12 $\pm$ 0.0 ^a	0.54 $\pm$ 0.0 ^a	3.66 $\pm$ 0.3 ^c	1.41 $\pm$ 0.0 ^a	0.60 $\pm$ 0.0 ^a	3.16 $\pm$ 0.2 ^d	1.31 $\pm$ 0.1 ^a	0.20 $\pm$ 0.0 ^a	3.33 $\pm$ 0.1 ^e	1.24 $\pm$ 0.1 ^a	0.19 $\pm$ 0.0 ^a	4.66 $\pm$ 0.3 ^d

444

445 Foot note. A: maximum colony diameter during stationary phase; v_m : maximum growth rate; λ : lag time

446 Values are means $\pm$ standard error. Within each column means with the same lowercase letter are not significantly different (P > 0.05).

447

448

449

450

Figure: 1 Effect of different EOs on the maximum colony diameter, $\ln(D_t/D_0)$, of *A. niger* over time.

Figure: 2 Effect of different EOs on the maximum colony diameter, $\ln(D_t/D_0)$, of *A. flavus* over time.

Figure: 3 Effect of different EOs on the maximum colony diameter, $\ln (D_t/D_0)$, of *A. parasiticus* over time.

Figure: 4 Effect of different EOs on the maximum colony diameter, $\ln(D_t/D_0)$, of *P. chrysogenum*.

Figure: 5 Effect of different EOs on the maximum colony diameter, $\ln (D_t/D_0)$, of *A. niger*, *A. flavus*, *A. parasiticus* and *P. chrysogenum* over time.